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Ambiguities in results obtained with 2D gel replicon mapping techniques

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ABSTRACT

Recently, two 2-dimensional (2D) gel techniques, termed neutral/neutral and neutral/alkaline, have been developed and employed to map replication origins in eukaryotic plasmids and chromosomal DNA (1–11). The neutral/neutral technique, which requires less DNA for analysis, has been preferentially used in recent studies. We show here that the signal predicted for an origin is not detected using the neutral/neutral technique if the origin is located near the end of the analyzed restriction fragment. We also demonstrate that analysis of the same batch of DNA by the two different mapping techniques can generate apparently contradictory results: in some situations where neutral/alkaline 2D analysis indicates that a certain origin is always used, neutral/neutral 2D analysis suggests that the origin is not always used. Several possible explanations for this type of disagreement between the two techniques are discussed, and we conclude that it is important to use both techniques in combination in order to minimize possible misinterpretations.

INTRODUCTION

The recent development of two independent 2-dimensional (2D) agarose gel replicon mapping techniques has permitted the first unambiguous mapping of eukaryotic replicons. The neutral/neutral 2D (N/N 2D) technique, developed by Brewer and Fangman (1,2), exploits the abnormal migration of non-linear structures under certain conditions in the second dimension. The second technique, developed in our lab (3,4,5), employs an alkaline second dimension to permit size analysis of nascent single strands of DNA and is therefore referred to as the neutral/alkaline 2D (N/A 2D) technique.

The two mapping techniques have proven to be powerful tools in the localization of replication origins in yeast plasmids (1,2,3,5), SV40 DNA (4) and in the replication analysis of yeast chromosomal DNA (6,7,8). Recently, the N/N 2D technique has been used to analyze replication during amplification of the chorion gene cluster in *Drosophila* (9). This technique was also used to locate a replication origin in an Epstein-Barr virus *oriP*-

containing plasmid (10) and to study the dihydrofolate reductase downstream origin in CHO 400 cells (11).

The N/N 2D technique has two practical advantages over the N/A 2D technique: it requires less DNA for a detectable signal, and a single hybridization usually suffices to reveal the replicative character of the restriction fragment of interest, i.e. whether it is replicated from the outside (as a simple 'Y' shape) or is replicated from an internal origin. In contrast, the N/A 2D technique requires hybridization with two or more shorter probes to determine the character of replication in the restriction fragment of interest and also requires more DNA since separation of nascent and parental strands distributes the target DNA over a greater surface area.

We have found, however, that N/N 2D analysis of a replication-origin-containing restriction fragment can sometimes fail to show initiation signals. There are two distinct ways in which an initiation signal can be lost. First, the location of the replication origin within a restriction fragment determines whether a characteristic 'bubble' arc, indicative of initiation at an internal origin, will be detected. Second, origin-containing restriction fragments from chromosomal DNA (as opposed to plasmid DNA) frequently give rise to a composite of 'bubble' and 'Y-like' signals even when N/A 2D analysis indicates that all replication initiates inside the fragment.

We report here our studies of these phenomena and explore their possible causes. We conclude that, because the two mapping techniques are independent and complementary, use of both techniques in combination can be helpful in eliminating interpretation ambiguities.

MATERIALS AND METHODS

Cell growth

Unsynchronized *S. cerevisiae* cells, strains CT711 (12; *leu2-3,-113, his3Δ1, trp1, ura3-52, ade2-101, can1*) and YPH3 (obtained from Robert Umek who received the strain from Philip Hieter, John Hopkins Univ.; *ura3-52, lys2-801, ade2-101*) were grown in YPD medium (13); occasionally YPH3 was grown in SD (Standard Defined; 13) medium supplemented with adenine sulfate (20 mg/l), L-lysine-HCl (30 mg/l) and uracil (20 mg/l). Starter cultures were routinely prepared from individual colonies

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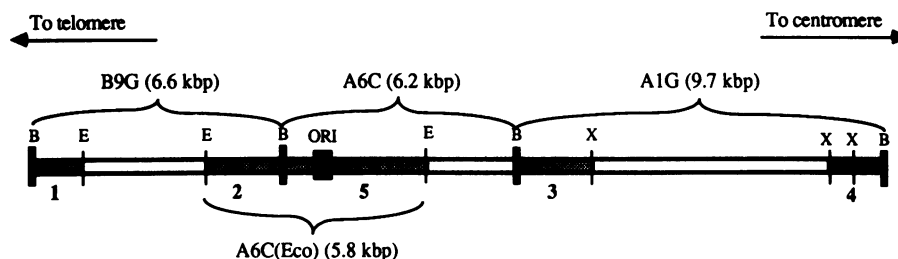


Figure 1. Map of the A6C region. Restriction sites are indicated (n.b. this is not a complete restriction map): B = *Bam*HI, E = *Eco*RI, X = *Xho*I. The analyzed fragments B9G, A6C, A6C(Eco) and A1G (see Fig. 3–5) are shown on the restriction map with their respective sizes indicated in kilobase pairs (kbp). The probes, shown as hatched boxes, are numbered 1–5. The replication origin is depicted as the black box on the restriction map. Directions to the telomere and centromere are indicated by the arrows.

of the various strains. The cells were grown at either 23°C or 30°C to a density of 2×10^7 cells/ml. Strain 4910-3-3 was grown as previously described (6).

DNA isolation

DNA was isolated using the glass beads method as described (3). In order to reduce the size of the chromosomal DNA before further isolation in one experiment nuclei from CT711 cells were digested with *Eco*RI (see results): 2.5×10^9 nuclei were pelleted after the glass bead treatment (3) and washed four times in 5 ml *Eco*RI restriction buffer (10 mM Tris/HCl pH 8.0, 0.15 mM spermine, 0.5 mM spermidine, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 mM NaCl). The nuclei were incubated with 1500 units *Eco*RI at 23°C for one hour in a 1 ml volume. This resulted in 25–50% digestion of the DNA as analyzed by agarose gel electrophoresis. The samples were then brought up to 10 ml with Proteinase K incubation buffer and the DNA isolation was continued as described (3) with Proteinase K incubation.

2D analysis of replication intermediates

All procedures were done as previously described. See refs. 1 and 2 for N/N 2D analysis; refs. 3, 4 and 5 for N/A 2D analysis; and refs. 6 and 7 for the use of both techniques combined. Preparation of the probes used in this paper is described in ref. 6. Restriction enzymes were obtained from Bethesda Research Laboratories. Various exposures of the autoradiograms were scanned with a 300A computing densitometer (Molecular Dynamics) which is capable of integrating signals over irregular areas. Background densities were determined from regions of the autoradiograms as near as possible to the measured regions.

RESULTS

Detection of initiation signals in N/N 2D analysis depends on the position of the origin

Replication of the extreme left arm of chromosome III in *S. cerevisiae* has been the subject of recent studies in our lab (6 and unpublished). Based on the use of both the N/N 2D and the N/A 2D techniques we concluded (6) that replication always initiates at the A6C origin and proceeds bidirectionally. Fig. 1 presents a map of the region immediately adjoining the A6C origin and indicates the probes and restriction sites used. The A6C origin is indicated as a black box. This origin is located near the center of the 5.8 kbp (kilobase pairs) *Eco*RI fragment A6C(Eco) and at the left-hand side of the 6.2 kbp *Bam*HI fragment A6C.

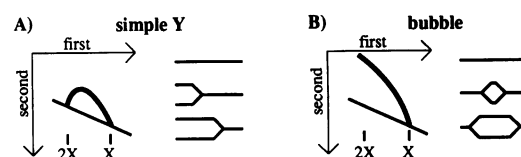


Figure 2. Illustration of typical arcs obtained in N/N 2D analysis. A) The left part shows the arc expected if a restriction fragment is replicated from one end to the other as a 'Y', as diagrammed in the cartoon on the right. B) The typical 'bubble' arc (left) is observed if a restriction fragment is replicated from within (right). See text for further details.

The main signals generated by replication intermediates in N/N 2D analysis are shown in Fig. 2. A restriction fragment that is replicated as a simple 'Y', from one end to the other, gives rise to a 'Y' arc as shown in Fig. 2A. A restriction fragment that is replicated from a centrally located internal origin will show a characteristic 'bubble' arc in N/N 2D analysis (Fig. 2B). If the origin is located asymmetrically in a restriction fragment one would expect that initially, from unreplicated to the point in replication where the 'bubble' becomes a 'Y', the signal would appear as a 'bubble' arc (Fig. 2B). From that point on to size $2 \times$ the signal would resemble a 'Y' arc (Fig. 2A, refs. 1, 7).

Figure 3 shows the results of hybridizing *Bam*HI- and *Eco*RI-digested *S. cerevisiae* 4910-3-3 DNA with probe 5 (Fig. 1). Hybridization with probe 5 detects the A6C(Eco) fragment in *Eco*RI-digested DNA and the A6C fragment in *Bam*HI-digested DNA (Fig. 1). A signal due to internal initiation, a so-called 'bubble' arc, is produced by the *Eco*RI fragment, which contains the origin near its center (Fig. 3, left). The additional signal in Fig. 3 (left), which resembles a 'Y'-arc, will be discussed later.

The A6C origin is 1.0 kbp (or 16% of fragment length) from the left end of the 6.2 kbp A6C *Bam*HI fragment (Fig. 3, right). Bidirectional replication from this origin is expected to produce replication bubbles during replication of the first third of this fragment. In the *Bam*HI digest, however, no evidence is seen of a 'bubble' arc, which would be expected from 6.2 kbp to 8.2 kbp in the first dimension (Fig. 3, right). In fact, the arc detected in the *Bam*HI digest is indistinguishable from the arc produced by the B9G fragment (Fig. 5 in reference 6), a fragment which does not contain an origin and is replicated as a simple 'Y'-structure from right to left (6). Similarly, N/N 2D analysis of an 8.6 kbp fragment which contains the C1G origin on chromosome III did not show a 'bubble' arc even though the origin was located 2 kbp (23% of fragment length) from the end of the fragment. As in the case of the A6C origin, a 'bubble' arc was observed if restriction sites were chosen that placed the

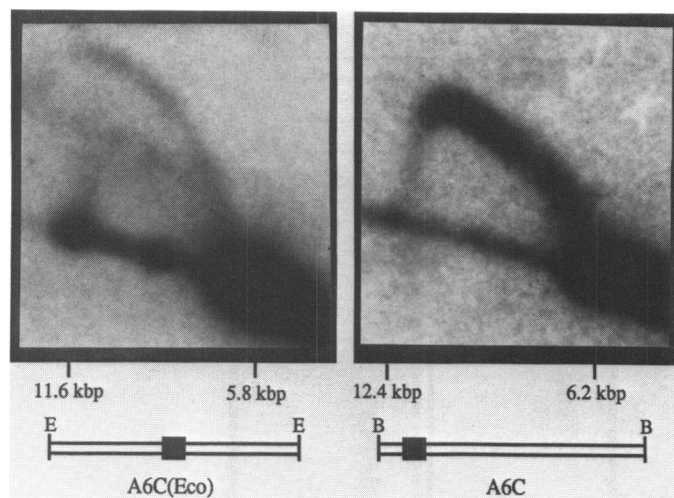


Figure 3. N/N 2D analysis of two restriction fragments containing the same origin differently located with respect to the ends of the restriction fragments. Left) An autoradiogram of replication intermediates detected by probe 5 (Fig. 1) in 4910-3-3 DNA digested with *EcoRI*. The fragment detected is A6C(Eco) (Fig. 1). Exposure time 7 days. The arc due to 'bubble' (initiating) structures is clearly present. The nature of the additional signal is discussed in the text and in ref. 6. Right) Replication intermediates detected by probe 5 in *BamHI*-digested 4910-3-3 DNA. Exposure time 6 days. The signal looks like a complete 'Y'-shaped arc (compare with B9G fragment in ref. 6), even in the first 1/3 of the arc (from 6.2 kbp to 8.2 kbp), where a signal due to replication initiation (higher 'bubble' arc) would be expected.

C1G origin in the middle of the resulting restriction fragment (J.Zhu, C.S. Newlon, and J.A. Huberman, manuscript in preparation). These observations suggest that, even when replication initiates within a restriction fragment, a 'bubble' arc is likely to be observed only if the origin is located within the central portion of the fragment. Apparently the N/N 2D signal generated by a small replication bubble located at the end of a restriction fragment is indistinguishable from the signal generated by a small 'Y' structure.

Composite Signals from Origin Regions in N/N 2D Analysis

In related studies of A6C region replication, several other yeast strains were analyzed. N/N 2D analysis of A6C(Eco) for strain YPH3 grown in SD medium at 23°C and for strain CT711 grown in YPD medium at 30°C are shown in Figures 4A and 4B respectively. In both cases composite patterns of 'bubble' and 'Y' arcs were observed. These observations helped us to realize that a weak 'Y' arc is also present in the N/N 2D analysis of A6C(Eco) of yeast strain 4910-3-3 (ref. 6 and Figure 3A). Initially, we did not interpret this signal in strain 4910-3-3 as 'Y'-like because the signal was weak and because the results of the N/A 2D technique appeared to indicate clearly that all detectable replication initiated at the A6C origin (6). However, further analyses indicated that the observation of two arcs in all these strains was highly reproducible (data not shown). We therefore sought an explanation for the two arcs.

The simplest hypothesis was that the two different arcs were generated by two classes of replication intermediates: replication bubbles due to internal initiation and 'Y'-shaped structures generated by replication of this restriction fragment from the outside (see Fig. 2). The appeal of this simple hypothesis led us to conclude prematurely that the A6C origin region in strains YPH3 and CT711 was sometimes replicated from an outside origin (ref. 14, page 3).

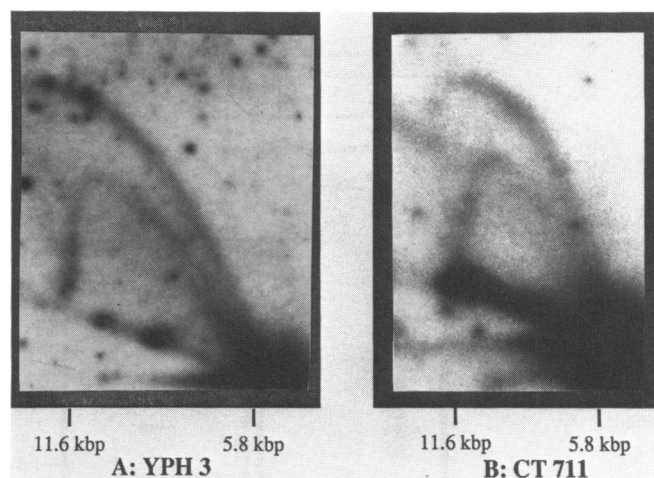


Figure 4. Composite signals obtained by N/N 2D analysis of A6C replication. A) Replication intermediates detected by probe 5 in YPH3 DNA digested with *EcoRI*. YPH3 cells were grown in SD medium at 23°C. Exposure time: 3 days. B) CT711 DNA digested with *EcoRI* and analysed with probe 5. CT711 cells were grown in YPD at 30°C. Exposure time: 3 days.

Evidence that this simple hypothesis was incorrect came from experiments designed to determine whether the 'Y'-shaped molecules were due to replication forks entering the A6C(Eco) fragment from the left or from the right. For these experiments the same DNA preparation was used that generated the composite pattern in Fig. 4B. We used a scanning densitometer capable of integrating signals over irregular areas to estimate the proportion of signal in the 'bubble' and 'Y'-like arcs in Fig. 4B and concluded that $\approx 43\%$ of the total signal from replicating structures is in the 'Y'-like arc. However, the replicating molecules responsible for the 'bubble' arc each contain two replication forks while those responsible for the 'Y'-like arc might contain only a single fork. If replication forks move at constant rates, then the 'bubble' molecules would complete replication twice as fast as the 'Y'-like molecules. Therefore, to account for the anticipated two-fold relative reduction in 'bubble' signal, we multiplied the 'bubble' signal by two to arrive at a final estimate of $27 \pm 7\%$ of total replicating molecules in the 'Y'-like arc.

In order to determine from which side the A6C region was replicated $27 \pm 7\%$ of the time (Fig. 4B), we used the independent N/A 2D technique to characterize replication of the two restriction fragments adjacent to the A6C fragment. The N/A 2D technique complements the N/N 2D technique because it can determine unambiguously in what direction a replication fork moves through a restriction fragment (3,4,5,6,7). In brief, 2D gel electrophoresis is used to separate nascent strands from parental and nonreplicating strands, and also to separate nascent strands according to size. Under the conditions used, the nascent strands form a diagonal arc. A hybridization probe from the end of a restriction fragment where replication forks enter detects nascent strands of all sizes, while a probe from the other end detects only the very longest nascent strands.

Figure 5 shows that in the telomere-proximal fragment, B9G, probe 2 detects nascent strands of all sizes (a complete diagonal nascent strand arc is visible) while probe 1 does not detect any but the longest nascent strands (no diagonal nascent strand arc is visible). By scanning various exposures of each autoradiogram in this experiment, and based on previous experiments (7), we calculate that we would easily be able to detect small nascent

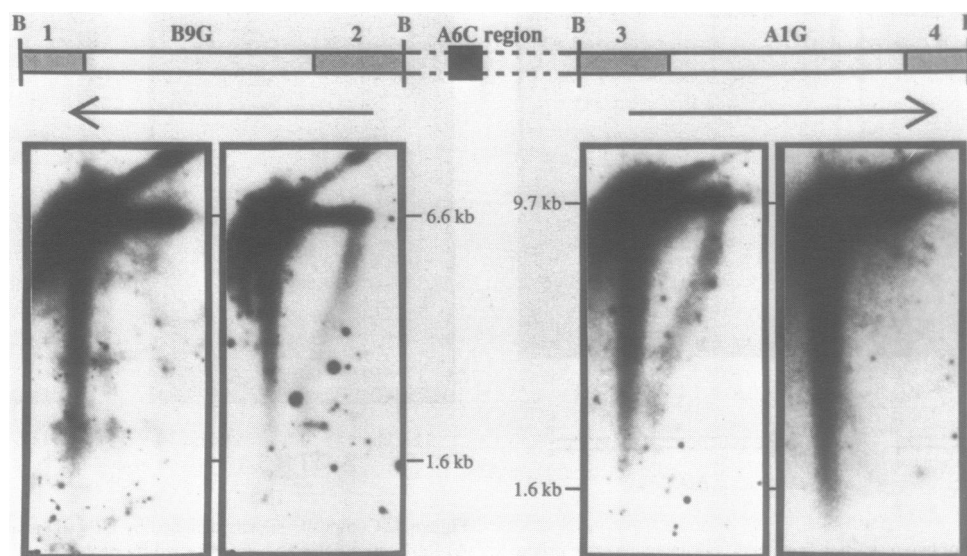


Figure 5. N/A 2D analysis of fragments B9G and A1G. CT711 DNA, from the same preparation that was used in Fig. 4B, was digested with *Bam*HI and analyzed with the N/A 2D technique. Each autoradiogram is the result of hybridization of the membrane with the probe that is shown above the autoradiogram in the map of the region. Each probe was hybridized to the same membrane, with subsequent stripping of the membrane. Probes 2 (in B9G) and 3 (in A1G) recognize nascent strands of all sizes (the diagonal smear), while probes 1 (in B9G) and 4 (in A1G) do not. The arrows in the diagram indicate the inferred direction of replication through the two fragments. The sizes in kilobases (kb) indicated to the sides of the autoradiograms are the sizes of single strands in the second dimension. Exposure times: panel 1, 5 days; panel 2, 1 day; panel 3, 4 days; panel 4, 5 days.

strands with probe 1 if the fragment were replicated from probe 1 towards probe 2 five percent or more of the time. This indicates that the B9G fragment is replicated from probe 2 towards probe 1 almost exclusively ($>95\%$) away from the A6C origin. Surprisingly, the same type of experiment shows that the fragment located on the centromere side of the A6C region, A1G, is also replicated away from the A6C origin $>95\%$ of the time (Fig. 5). If the 'Y'-like arc in the N/N 2D analysis of A6C(Eco) (Fig. 4B) were indeed due to replication from the outside $27 \pm 7\%$ of the time we should have detected nascent strands of all sizes with probe 1 (in B9G) or with probe 4 (in A1G). Even if outside replication into the A6C region could originate simultaneously from both sides, the estimated maximum amount of undetectable small nascent strands would be $<10\%$ ($2 \times <5\%$, for probes 1 and 4 combined). Thus the results of the N/A 2D analysis are incompatible with the interpretation that the A6C region is replicated from the outside $27 \pm 7\%$ of the time and suggest that the A6C origin is used $>90\%$ of the time, perhaps all the time. Since we cannot easily explain why the N/A 2D technique would fail to detect replication from the outside into the A6C region, we propose that the 'Y'-like arc in Fig. 4B is not due to replication of the A6C region from the outside and does not represent replication of this fragment by 'Y'-shaped replication intermediates having daughter arms of equal lengths.

Possible Causes for a 'Y'-Like Arc

A restriction fragment containing an internal bubble could produce a 'Y'-like arc if a parental strand break were to occur in either of the single-stranded regions at either of the two replication forks of the bubble. During N/N 2D electrophoresis, the resulting singly-branched structures would migrate similarly to molecules that are 'Y'-shaped due to replication from one end. Between 30% and 50% replication, the broken structures would be maximally nonlinear and would exhibit maximally retarded migration. As the broken structures approached 100% replication,

they would (like true 'Y'-shaped molecules) increasingly resemble linear molecules and would migrate more rapidly. The 'Y'-like arcs in Fig. 4 could be caused by such parental strand breaks.

One possible cause for single-strand breaks in replication intermediates might be a single-strand nuclease which could be present in restriction enzyme preparations. We therefore tested the enzymes used in this study for single-strand nuclease contamination. Although some restriction enzymes do contain considerable single-strand nuclease activity, the enzymes used in this study did not show any detectable activity (data not shown).

It is notable that in the N/N 2D gel analysis of origin-containing restriction fragments derived from plasmid (as opposed to chromosomal) DNA, 'Y'-like arcs were not obvious (although sometimes a very faint 'Y' arc could be detected; 1). Thus, 'Y'-like arcs produced by origin-containing restriction fragments seem to be relatively specific for chromosomal DNA. It seemed possible that, during the isolation of high molecular weight DNA, cumulative shear forces on the long DNA strands could generate a large pulling force at replication bubbles, thereby breaking bubbles at the fork or within the bubble itself. In order to test this idea, we modified the DNA isolation procedure so that the DNA would be cleaved (at specific sites) into relatively short pieces (which would be more resistant to shear) prior to decondensation by removal of histones. After isolation of nuclei, one half of the nuclei was incubated with *Eco*RI (see Materials and Methods). The incubation was performed at 23°C in order to reduce possible endogenous endonuclease activity. Under our conditions 25–50% of the *Eco*RI sites in intact nuclei were cleaved. The average size of the resulting DNA fragments was 10–20 kbp, as analyzed by conventional agarose gel electrophoresis (data not shown). DNA was then isolated from both the digested and the non-digested nuclei as usual (see Materials and Methods). After isolation, both samples were digested to completion with *Eco*RI and the samples were subjected to N/N 2D analysis. In both the control and the 'digested nuclei'

samples 'Y'-like arcs were observed similar to the arcs in Fig. 4 (data not shown). There was no difference between the two samples in relative strength of the 'Y'-like arcs, suggesting that pulling on long DNA molecules during DNA isolation is not the cause of 'Y'-like arcs.

The ratio of the intensity of the 'Y'-like arc to the intensity of the 'bubble' arc appeared to be relatively constant for a given cell strain. The 'Y'-like arc for the A6C origin is weak in the 4910-3-3 strain. N/N 2D mapping of the C1G origin in 4910-3-3 also did not reveal a clear 'Y'-like signal (J. Zhu, C.S. Newlon, and J.A. Huberman, manuscript in preparation). In the strains CT711 and YPH3, the ratio appeared to be consistently higher than in 4910-3-3, and independent of growth conditions such as media and temperature (data not shown). This consistency also argues against the idea that shearing or nuclease action during DNA isolation and preparation (which would be expected to vary from experiment to experiment) can cause conversion of 'bubble' structures into 'Y'-like structures.

If the 'Y'-like arcs were due to the introduction of breaks into parental strands at replication forks, then two families of nicked parental strands would be generated from A6C(Eco) fragments of increasing extents of replication, and these families would produce diagonal signals in predictable locations after N/A 2D gel electrophoresis. To test this possibility we carried out several N/A and N/N 2D gel analyses of the A6C(Eco) fragment using DNA from both CT711 and YPH3 cells. The N/N analyses all produced clear composite arcs similar to those in Fig. 4. However, the N/A analyses all produced high background in the regions predicted to contain diagonal signals from specifically nicked parental strands. Although these negative results do not permit any firm conclusions to be drawn, the high background suggests that parental strands near the A6C origin may be nicked at multiple locations.

DISCUSSION

During the course of our investigations of DNA replication patterns in *S. cerevisiae*, we observed that using the N/A 2D technique and the N/N 2D technique for analysis of the same region could sometimes lead to apparently contradictory signals. We demonstrate here that it is possible to misinterpret the replication pattern if one uses only a single technique.

In N/N 2D analysis a 'bubble' arc, characteristic of initiation of replication, is not seen if an origin is located 16% (Fig. 3) or 23% (J. Zhu, C.S. Newlon, and J.A. Huberman, manuscript in preparation) of fragment length from the end of a restriction fragment. However, when an origin is located as far as 33% from a restriction fragment end, then a partial 'bubble' arc is easily detected (ref. 1, Fig. 7A). These observations show that by using only the N/N 2D technique for mapping origins in a large chromosomal region one might easily miss an origin if the origin is located too close to a restriction site. Use of various restriction enzymes can partly overcome this problem, but sometimes it is difficult to obtain several different restriction digests yielding fragments of suitable size (3–10 kbp) for analysis of a particular region. The N/A 2D technique detects the direction(s) of replication in each restriction fragment, thus permitting at least crude origin localization even when favorable restriction sites are not available.

The results of N/N 2D analysis (Fig. 4) and N/A 2D analysis (Fig. 5) of the A6C region appear to be contradictory. We think that the 'Y'-like arcs in Fig. 4 are not due to 'Y'-shaped

replication of the A6C origin region from an external origin because N/A 2D analysis did not detect any evidence for external replication of the A6C origin, and we can see no reason why the N/A 2D technique would not yield a detectable signal if external replication were >10% of total replication. The N/A 2D technique succeeded in detecting replication occurring in the opposite direction at a level of about 5% in yeast rDNA (7). In the case analyzed here, external replication would have to be $27 \pm 7\%$ of total replication to account for the intensity of the 'Y'-like arc in Fig. 4B.

An alternative explanation for 'Y'-like arcs like those in Figure 4 would be the presence of a break at a replication fork in one of the parental strands of the replication bubble. We tested two possible causes for such putative breaks and neither single-strand nuclease contaminating the restriction enzymes nor shearing during isolation of the DNA seemed likely explanations. The facts that the 'Y'-like arc is observed mostly in chromosomal DNA and that its intensity within one strain appears relatively constant also argue against artifacts such as shearing or nuclease activity during isolation. A direct attempt to detect such putative breaks by N/A 2D gel electrophoresis gave unclear results.

Other groups have also observed this composite pattern of 'bubble' and 'Y'-like arcs in origin regions. The composite pattern was consistently detected during analysis of origin-containing fragments from the chorion gene cluster of *Drosophila melanogaster* (9). Likewise, during analysis of a restriction fragment containing the Epstein Barr virus *oriP* (10), a faint 'Y' arc was observed in addition to a 'bubble' arc. The authors attributed this signal to 'Y'-shaped replication of the origin region in oligomers. However, oligomers were estimated to represent less than 1% of the plasmids (10), but the intensity of the 'Y' signal appeared considerably greater than 1%. 'Y'-like signals were also observed during N/N 2D analysis of SV40 DNA (R. Kelly, P. DeRose and G. Wahl, personal communication). Other investigators noted that the major signal detected during N/N 2D analysis of restriction fragments from the dihydrofolate reductase downstream origin region in CHO 400 cells was a 'Y'-like arc (11) even though other mapping techniques show that replication origins are present in the tested region (15, 16, 17). 'Bubble' arcs from this origin region could be detected only with difficulty (11).

During N/N 2D analysis of rDNA in *S. cerevisiae* (7) we also observed a composite of 'Y' and 'bubble' arcs. The 'Y' arc was always strong and the 'bubble' arc was always weak. We interpreted the ratio of the intensities as an indication of the frequency of origin usage. This interpretation, that each rDNA origin region can be replicated either from its own origin or, more frequently, from a nearby origin, was, in this case, supported by N/A 2D analysis (7).

In a similar study of *S. cerevisiae* rDNA replication, Brewer and Fangman (8) detected only 'Y' arcs from origin-containing restriction fragments. To account for the absence of detectable 'bubble' arcs, they concluded that initiation must occur at less than one in 5 origins (8). The reason why we saw a weak 'bubble' arc while Brewer and Fangman did not might be that different strains were used. Our initial studies (7) were performed with strain 4910-3-3, which seems less likely than other strains to produce an obvious 'Y'-like arc (this paper). We failed to detect 'bubble' arcs at the rDNA origin in strain YPH3 and could barely detect a 'bubble' signal in strain CT711 (data not shown). It is possible that the strain used by Brewer and Fangman (8) resembles strains YPH3 and CT711 and produces a significant amount of a 'Y'-like arc.

The presence of a 'Y'-like arc could well reflect an in vivo state of the DNA. For instance, if topoisomerases act at the replication bubble, the isolation procedure might preserve their action as breaks in the bubble. Alternatively, it is possible that initiation of chromosomal replication may occur over a larger region than previously guessed, perhaps over many kbp. The resulting population of replication intermediates of an origin-containing restriction fragment would be a mixture of 'bubble'- and 'Y'-shaped molecules. In this case however, one would expect a more intense early portion (0% to $\approx 50\%$ replication) of the 'bubble' arc, and a more intense late portion ($\approx 50\%$ to 100% replication) of the 'Y' arc, because restriction fragments with asymmetrically located initiation sites would produce 'bubble' signals during early replication and would be converted to 'Y' structures during later replication. This prediction is not fulfilled in our experiments (Figs. 3 and 4).

Provision of a complete explanation of 'Y'-like arcs generated by origin-containing restriction fragments falls outside the scope of this paper. Here we simply wished to demonstrate possible difficulties in interpretation that can arise when only one 2D gel replicon mapping technique is used. The two available 2D gel techniques are independent and complementary and are, when used in combination, a very powerful tool for analysis of DNA replication.

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